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Therapeutic Antisense Oligonucleotides Are Coming of Age

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Abstract

The first published description of therapeutic applications of antisense oligonucleotide (ASO) technology occurred in the late 1970s and was followed by the founding of commercial companies focused on developing antisense therapeutics in the late 1980s. Since the late 1980s, there has been steady progress in improving the technology platform, taking advantage of advances in oligonucleotide chemistry and formulations as well as increased understanding of the distribution and safety of ASOs. There are several approved ASO drugs and a broad pipeline in development. In addition, advances in understanding human disease, including the genetic basis for most monogenic diseases and the availability of the full human genome sequence, have created numerous therapeutic applications for the technology. I summarize the state of the technology and highlight how advances in the technology position ASOs to be an important contributor to future medicines.

INTRODUCTION

Over the past century, the pharmaceutical and biotechnology industries have successfully developed methods and strategies to therapeutically target proteins. Although serendipity still plays a role in traditional drug discovery, the science behind small-molecule and protein-based drug discovery has significantly matured over the past 25 years. The issue is that only a subset of targets that have been linked to human diseases are directly approachable with small-molecule or protein-based therapeutics. To address the broad therapeutic needs of patients, it is critical to diversify the targets that can be therapeutically approached. RNA represents one class of targets.

As proteins are derived from specific mRNAs, modulating mRNA or pre-mRNA levels could be used to broaden the set of therapeutic targets. Proteins that are difficult to target using conventional small-molecule or protein-based strategies (adapter proteins, transcription factors, etc.) can be readily targeted by modulating the mRNA levels and/or translation to the protein. In addition, identification of numerous classes of noncoding RNAs and knowledge of how they regulate normal cell physiology are expanding. Several unique regulatory roles of noncoding RNAs have been identified (1–3), and it has been demonstrated that RNAs can directly promote pathology (4). Currently, several different therapeutic strategies are employed to modulate RNA function in cells. These include small molecules targeting RNA, gene therapy, genome editing, delivery of exogenously expressed mRNAs, and synthetic antisense oligonucleotides (ASOs). This review focuses on antisense oligonucleotide technology.

While antisense technology has gone through the different phases of technology development, from unbridled enthusiasm to abandonment because of challenges and failures and then to rebirth, there has been steady progress in advancing the technology. The recent approvals of several antisense drugs with numerous drugs in late-stage clinical studies provide optimism that antisense technology is delivering on its promise (5).

ANTISENSE OLIGONUCLEOTIDES: MOLECULAR MECHANISMS

For the purposes of this review, antisense oligonucleotides (ASOs) are defined as chemically synthesized oligonucleotides, generally 12–30 nucleotides in length, that are designed to bind to RNA by Watson-Crick base pairing rules (**Figure 1**). The length of ASOs in part contributes to their specificity, as oligonucleotides that are 16–20 nucleotides long are capable of uniquely binding to only one target RNA. Following binding to the targeted RNA, the oligonucleotide modulates RNA function by several different mechanisms. These can be broadly categorized as mechanisms promoting RNA cleavage and degradation or occupancy-only mechanisms, sometimes referred to as steric blocking (**Figure 2**). The mechanism(s) by which the ASO modulates the RNA is dependent on the ASO chemistry and design, the position on the RNA where the ASO is designed to bind, and the function of the RNA. Based on the chemical and positional requirements for the different mechanisms, it is possible to rationally design ASOs to modulate the target RNA, although some screening is still required for optimal activity and tolerability.

Occupancy-Only Mechanisms

Several antisense mechanisms do not result in direct degradation of the target RNA (**Figure 2*a***) (6, 7). Paul Zamecnik is credited for first introducing the concept that synthetic oligonucleotides could be developed therapeutically to block protein translation (8). His seminal papers demonstrated that a synthetic oligodeoxynucleotide designed to bind to the Rous sarcoma virus RNA blocked translation of the viral RNA and subsequently blocked virus replication (9, 10). Once

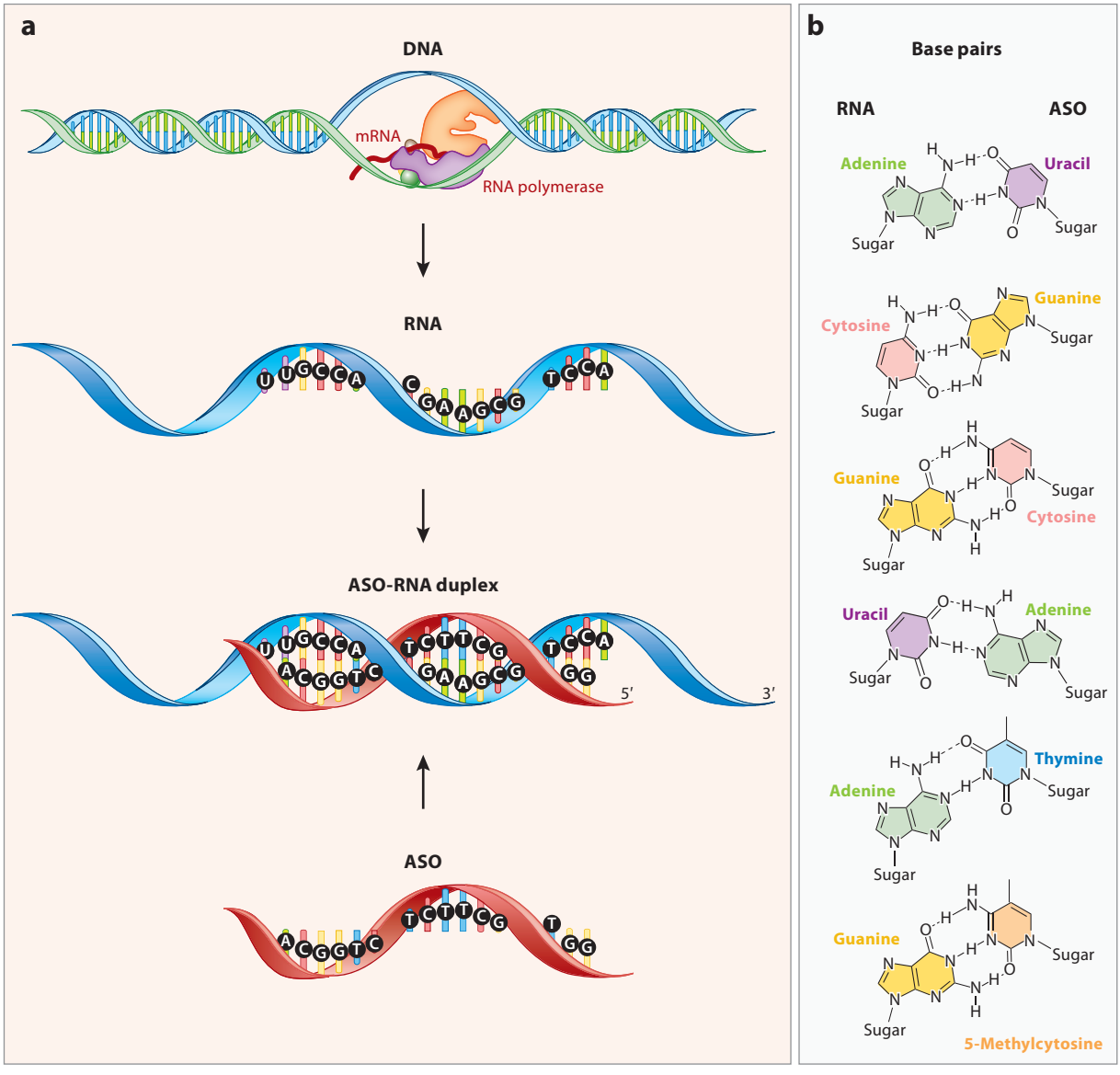

Figure 1

Antisense oligonucleotide (ASO) binding to the targeted RNA. (*a*) The RNA polymerase transcribes the RNA from the DNA template. The synthetic ASO (*red*) binds to the RNA (*blue*) by Watson-Crick base pairing rules, e.g., adenine binds to uracil and cytosine binds to guanine. (*b*) The modified bases thymine and 5-methylcytosine are frequently used in ASO drugs.

the technology for chemical synthesis of oligonucleotides was developed (11), interest in using oligonucleotides as a therapeutic platform expanded. Blocking protein translation remains a viable antisense mechanism, but it is not broadly used as a therapeutic strategy. More recently, several approaches to increase protein translation have been published.

MicroRNAs are short RNAs (approximately 21 to 23 nucleotides) that repress translation of multiple mRNAs targets, resulting in control of gene networks (3). ASOs designed to bind to

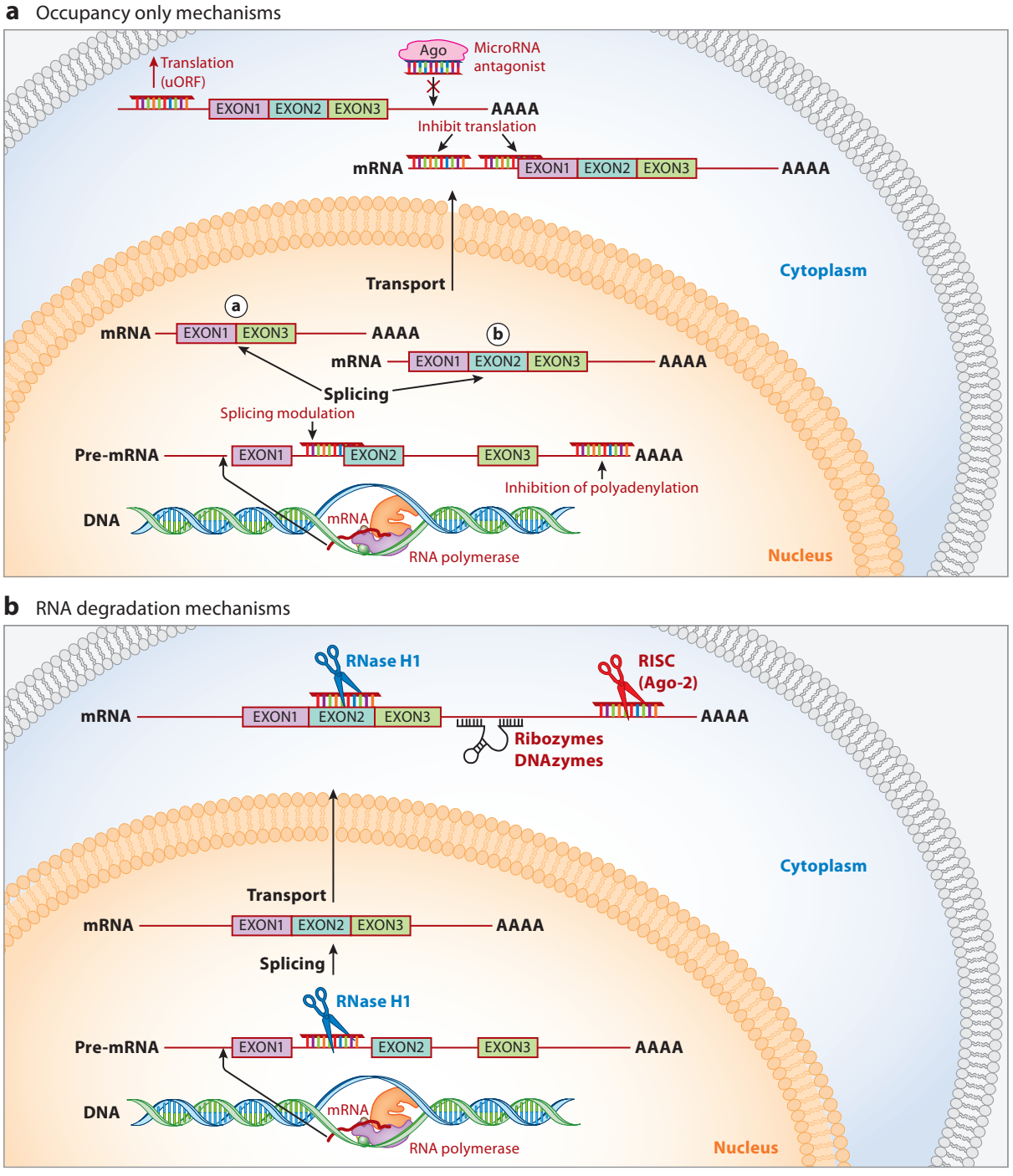

Figure 2

Different mechanisms of action for antisense oligonucleotides. (*a*) Occupancy-only antisense mechanisms do not result in degradation of the targeted RNA. Abbreviation: uORF, upstream open reading frame. (*b*) RNA cleavage antisense mechanisms promote degradation of the targeted RNA.

microRNAs block their ability to bind to targeted RNA sequences, resulting in de-repression of translation of the microRNA targets (12, 13). Because microRNAs block translation of multiple targets, often in a tissue- or cell-specific manner, blocking a single microRNA results in increased expression of numerous proteins. A more specific approach to increase protein production is to design an ASO to bind to a regulatory sequence in the 5′-untranslated region of a mRNA that represses protein translation, such as an upstream open reading frame or stem-loop structure (14, 15).

Most mammalian protein coding RNAs undergo a complex set of processing events that includes adding a 5′-cap structure, removing large segments of RNA sequence and splicing the RNA back together, and adding a polyadenylate (polyA) tail to the 3′-end of the RNA (1). Each of these steps can be selectively modulated by ASOs (**Figure 2*a***), with modulation of RNA splicing by ASOs being the most broadly utilized. ASOs can be designed to cause exon skipping, as is the case with eteplirsen (16), or to promote exon inclusion, as is the case for nusinersen (17), two recently approved antisense drugs. Additional therapeutic applications for modulation of RNA splicing are being explored in the laboratory and early clinical trials (5). Many transcripts have two or more alternate polyA sites that may be preferentially utilized in a disease state such as cancer (18). In addition, polyA site selection can mediate subcellular localization of an RNA transcript (19). ASOs have been shown to redirect which polyA site is utilized. Yet another example of using ASOs to modulate gene expression in cells is preventing long noncoding RNAs from interacting with their sites on chromatin, resulting in increased transcription of a repressed gene, as has been described for a long noncoding RNA that inhibits SMN2 gene transcription through recruitment of the PRC2 complex (20). As we enhance our understanding of the different regulatory roles RNAs play in health and disease, there will likely be additional mechanistic insights for the application of ASOs.

RNA Degradation Mechanisms

The majority of ASOs in development are designed to promote RNA cleavage by either RNase H1 or argonaute 2 (Ago2) (**Figure 2*b***) (5). RNase H1 is an endogenous nuclease present in most, if not all, cells, which promotes cleavage of the RNA in an RNA-DNA heteroduplex (21, 22). In mammalian cells, RNase H1 is found in the nucleus, mitochondria, and cytoplasm, where it serves several functions, including removing the RNA present in the Okazaki fragment, DNA repair, and resolution of R loops (22–24). Oligonucleotides that are designed to utilize RNase H1 as their mechanism of action must contain a minimum of 5 consecutive DNA nucleotides, with 7–10 being optimal. Oligonucleotides designed to degrade target RNA by the RNase mechanism are widely used as experimental tools and are being developed for a number of therapeutic indications (5). ASOs designed to work through the RNA interference pathway, e.g., small interfering RNAs (siRNAs), are also broadly used as experimental tools to selectively reduce the expression of a target RNA and also as potential therapeutic agents, with an increasing number of drugs entering clinical development (5). ASOs that work through the RNA interference pathway are generally delivered to the cell or organism as a duplex of two RNAs or modified RNAs, with one strand designed to bind to the target RNA and the second, or passenger, strand ultimately degraded (25, 26). Once inside the cytoplasm, the duplex binds to the nuclease Ago2 and releases the passenger strand (27, 28). The mechanisms by which Ago2 determines which strand to bind appear to be, in part, mediated by the 5′-end of the oligonucleotide, as well as the end with the least stable base pairing (29, 30). Like RNase H1, Ago2 has specific structural requirements for the oligonucleotide, limiting the types of chemical modifications that can be used. A key difference between these two mechanisms is that oligonucleotides that work through the RNase H1 mechanism bind to the target RNA before the enzyme is recruited, while siRNAs bind to the enzyme first and then the enzyme-oligonucleotide duplex binds to the RNA, although the former has not been conclusively proven.

The observation that RNA has the potential to exhibit enzymatic activity was a revolutionary discovery (31, 32) that has been exploited as a therapeutic antisense mechanism. Such catalytic RNAs and more recently DNAs (33) are referred to as ribozymes or DNAzymes. With the discovery of RNA interference and other antisense mechanisms, interest in ribozymes as a therapeutic strategy has waned. Other nucleases present in cells that in principle could be exploited to directly or indirectly promote the degradation of a target RNA are discussed elsewhere (34, 35).

OLIGONUCLEOTIDE CHEMISTRY AND FORMULATIONS

Unmodified RNA or DNA oligonucleotides are rapidly degraded in biological matrices and thus have limited utility as therapeutic agents. To enhance the drug-like properties of oligonucleotides, both chemical modifications and formulations are utilized. **Figure 3** shows the structures and key attributes of the different oligonucleotide chemical modifications currently used in clinical studies. For a detailed description of the various chemical modifications and formulations used for oligonucleotide drugs, the reader is referred to other recent reviews (36–39).

PHARMACOKINETICS

The pharmacokinetics of an ASO depends on whether it is single stranded or double stranded, whether it is negatively charged or neutral, and whether it is incorporated into a particulate formulation. Unformulated ASOs are small enough to be rapidly filtered out of blood by the kidney and excreted in urine. The pharmacokinetics of single-stranded phosphorothioate (PS)-modified oligonucleotides is well characterized (40). They exhibit increased binding to serum proteins such as albumin, which maintains them in circulation long enough to distribute to tissue. Following intravenous or subcutaneous administration, the phase of distribution from plasma to tissues ranges from minutes to a few hours followed by a prolonged elimination phase that can last for several weeks (40). The tissue distribution of PS-modified oligonucleotides is similar following subcutaneous or intravenous dosing; they distribute broadly to most peripheral tissues, with kidney and liver accumulating the highest concentrations. Following intrathecal administration, they appear to distribute within the cerebrospinal fluid (CSF) and from CSF to spinal cord and brain tissues, with highest drug concentrations observed in spinal cord and cortical tissue and less drug found in deeper brain structures (40, 41).

Our understanding of the mechanism(s) by which PS-modified ASOs accumulate in cells and ultimately distribute to the target RNAs is still rudimentary. Most of the published data suggest that PS-modified oligonucleotides bind to cell surface proteins and are subsequently endocytosed into intracellular vesicles (42). The ASOs ultimately escape from the membrane-bound organelles and likely bind to proteins that help shuttle them into the nucleus and perhaps facilitate hybridization to cytoplasmic and nuclear RNAs (43). PS-modified DNA ASOs have a relatively short tissue half-life of 2–3 days, while oligonucleotides containing both PS and sugar modifications such as 2′-O-methoxyethyl (MOE) have tissue half-lives measured in weeks (40).

Single-stranded neutral-backbone oligonucleotides such as morpholinos exhibit low serum protein binding and are rapidly filtered by the kidney (44, 45). Because of this rapid clearance, various conjugates are being explored to enhance the distribution and cellular uptake of morpholino ASOs, one of which is just starting clinical trials as a next-generation therapy for Duchenne muscular dystrophy (DMD). Double-stranded RNA ASOs are also rapidly cleared by the kidney and exhibit limited tissue distribution. Historically, lipid nanoparticle formulations have been used to enhance delivery of siRNA drugs to target tissues. As there are unique differences dividing the nanoparticle formulations, it is difficult to provide broad generalizations. Patisiran, a lipid

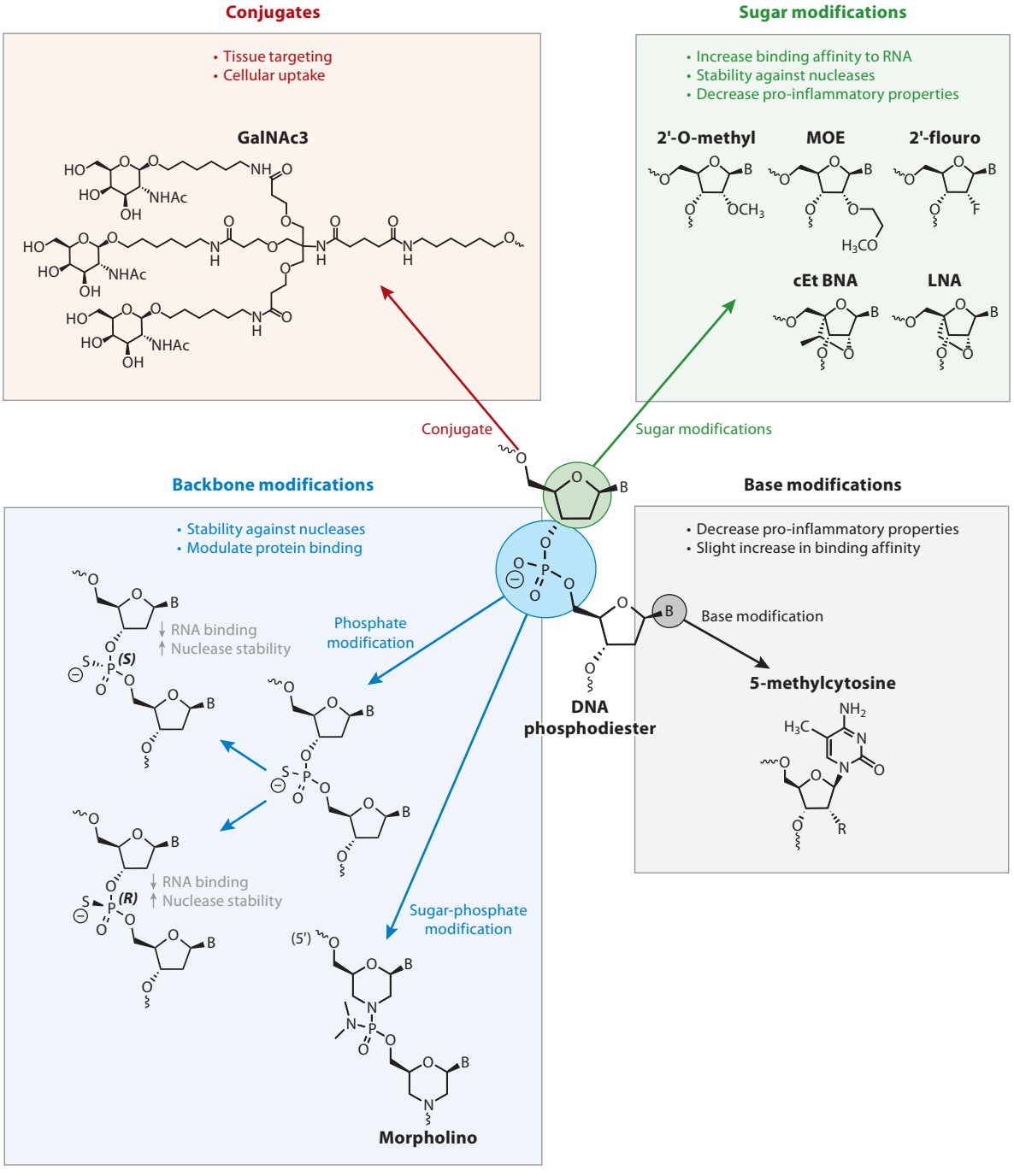

Figure 3

Common chemical modifications used in antisense drugs and their properties. A dinucleotide structure is shown, identifying positions where oligonucleotides are commonly modified. These positions include the phosphate backbone, the 2′ position on the sugar, the 5 position of pyrimidine bases, and addition of targeting ligands such as GalNAc sugars. Abbreviations: cEt BNA, (*S*)-constrained ethyl bicyclic nucleic acid; LNA, locked nucleic acid; MOE, 2′-O-methoxyethyl.

nanoparticle formulation of a transthyretin-targeting siRNA, exhibited dose-proportional plasma exposures, which correlated with reduction in plasma transthyretin (TTR) levels (46). Ionizable cationic lipid containing nanoparticles, such as the one used for the patisiran formulation, preferentially accumulate in hepatocytes, in part by association with ApoE particles in the circulation and by clearance by LDL receptors (47). The mechanism(s) by which the siRNAs escape from intracellular vesicles is not well characterized and warrants further investigation.

CLINICAL PHARMACOLOGY OF ANTISENSE DRUGS

Six antisense drugs have received market authorization by regulatory authorities, and at least four drugs are in phase III clinical trials or submitted for market authorization (5). A comprehensive review of the drugs in development is beyond the scope of this article. Instead, I discuss four examples that cover the spectrum of marketed or late-stage antisense drugs with respect to antisense mechanisms, chemistry, formulation, and target tissues. All four examples have been recently approved and involve rare genetic diseases that previously did not have approved therapies, highlighting the potential for RNA-targeted therapeutics to provide benefit to patients with untreatable conditions.

Inotersen

Inotersen is a second-generation antisense drug that prevents production of the transthyretin (TTR) protein by an RNase H1 dependent mechanism (48). TTR protein, which is primarily produced in the liver, forms a tetramer that binds retinal binding protein 4 (RBP4)-retinal complex, preventing renal clearance as well as serving as one of several thyroid hormone transport proteins (49). Autosomal dominant mutations in the *transthyretin* gene cause the tetrameric form of the protein to become less stable. The monomers form aggregates that deposit in multiple tissues including peripheral nerves, cardiac tissue, and kidney (49, 50). The aggregates usually lead to a peripheral neuropathy, severe gastrointestinal dysfunction, and in some cases cardiomyopathy. The average life expectancy of individuals with hereditary TTR (hTTR) is typically 3–15 years from symptom onset. Inotersen is a gapmer design with five 2′-MOE nucleotides on the 5′- and 3′-ends of the oligonucleotide and ten DNA nucleotides in the middle to support the RNase H1 mechanism. Inotersen produces a dose-dependent reduction of TTR mRNA and protein in cultured cells and in transgenic mice (48). In the transgenic mice, a single dose of inotersen produced effects that lasted 2–3 weeks. In cynomolgus monkeys, the inotersen binding site is complementary to the TTR sequence, allowing measurement of pharmacology. Monkeys administered inotersen demonstrated 90% reduction of TTR RNA expression in the liver (the main source of circulating TTR protein) and 80% reduction in circulating TTR protein (48). Treatment was well tolerated, with no deleterious liver or kidney effects observed.

A single and multiple ascending dose phase I clinical study was conducted in healthy volunteers (48). In the multiple-dose study, subjects were assigned to receive 0, 50, 100, 200, 300, or 400 mg of inotersen administered subcutaneously on days 1, 3, 5, 8, 15, and 22. The plasma TTR concentrations were stable in the placebo group throughout the duration of the study, and the mean percent reduction in plasma TTR ranged from 8% (50 mg dose group) to 76% (400 mg dose group) with no difference between the 300 and 400 mg dose groups. The drug effects lasted more than 30 days after dosing was stopped, consistent with the long half-life of the drug in liver tissue. No serious adverse effects occurred in the study, with 95% of adverse events considered mild. The most common adverse events were somnolence (33%) in single-dose subjects and injection-site reactions (4% of injections), increases in C-reactive protein (41%), headache (18%), vitamin A

decrease (18%), creatine phosphokinase increase (13%), and myalgia (10%) in multiple-dose subjects (48). Based on the efficacy and safety profile of Inotersen, a randomized double-blind placebo-controlled phase III trial was initiated in stage 1 and stage 2 patients with hereditary transthyretin amyloidosis (hATTR) polyneuropathy (NEURO-TTR, NCT01737398). One hundred seventy-two subjects were randomized to receive either weekly subcutaneous 300 mg injections of inotersen or placebo (2:1) over 15 months. The two primary endpoints were change in the modified Neuropathy Impairment Score+7 (mNIS+7) and change in the results of the Norfolk Quality of Life Diabetic Neuropathy questionnaire (51). Both primary efficacy endpoints were positive for inotersen-treated subjects compared to placebo. The least-squares mean change from baseline to week 66 between treatment arms was −19.7 points for the mNIS+7 and −11.7 points for the Norfolk Quality of Life questionnaire (51).

There were five deaths during the study, all in the inotersen group, four of which were consistent with progression or complication of the underlying disease. One subject in the inotersen arm had a fatal intracranial hemorrhage associated with grade 4 thrombocytopenia. Severe platelet declines below 25×10^3 platelets/μL occurred in two additional subjects in the inotersen arm, which returned to normal values following drug discontinuation and treatment with glucocorticoids. In response to these severe thrombocytopenia cases, the sponsor instituted weekly platelet monitoring, with no additional cases detected. In addition to these cases of severe thrombocytopenia, a higher proportion of subjects in the inotersen arm (54%) had confirmed decreases in platelet counts below 140×10^3/μL compared to placebo (13%). The mechanism(s) of decreased platelet counts following inotersen treatment are unknown but do not appear to be a class effect of MOE modified oligonucleotides (52). Three cases of glomerulonephritis occurred in patients treated with inotersen. The remaining adverse events were similar to those reported in the phase I study; however, a lower rate of injection-site reactions was observed in this study (1.1% of all injections). The phase III study clearly demonstrated a clinical benefit in the hATTR polyneuropathy patients, with the main safety issues being thrombocytopenia and glomerulonephritis, which can be managed by frequent monitoring. Based on these data, market authorization applications were submitted in both the United States and Europe. At the time of this writing, market authorization has been granted in the European Union and in the United States.

Patisiran

Patisiran is a chemically modified (eleven 2′-O-methoxy sugars) siRNA formulated in a lipid nanoparticle formulation, which also targets the TTR mRNA (46, 53). In a phase I study, a single dose of patisiran (ALN-TTR02), given as an intravenous infusion, produced dose-dependent reduction in plasma TTR protein with significant reduction in TTR observed at doses of 0.15, 0.3, and 0.5 mg/kg (46). Maximal reduction (>80% geometric mean) occurred ∼7 days after the infusion, and the effects lasted for more than one month (46). A moderate infusion-related reaction was observed in the one patient infused at a dose of 0.5 mg/kg. This study was followed by a phase II study in hTTR polyneuropathy patients (53). Subjects received two doses of patisiran by intravenous infusions 3–4 weeks apart. A reduction of >80% in plasma TTR protein was achieved at the highest dose tested (0.3 mg/kg every 3 weeks) and, similar to the phase I experience, the effects lasted more than 30 days after the last dose. Patients were pretreated with glucocorticoids, acetaminophen, and antihistamines to minimize the potential for infusion reactions. The most common adverse events were mild to moderate infusion reactions, which occurred in the 0.3 mg/kg dose group (∼40%). Starting with a slower rate of infusion over the first 15 min appeared to decrease the incidence of infusion reactions (53). Transient increases in white blood cell counts,

complement split products, and some cytokines and chemokines were observed 24 h after infusion in some patients but were not correlated with reported adverse effects.

A randomized double-blind placebo-controlled phase III study was recently completed in familial hTTR polyneuropathy patients (APOLLO II; NCT01960348) (54). Two hundred twenty-five patients were infused with either 0.3 mg/kg patisiran or placebo (2:1) every three weeks for 18 months, using the pretreatments and slow initial infusions to enhance tolerability. The primary endpoint of the study was mNIS+7, with secondary endpoints including quality of life, muscle strength, gait speed, nutritional status, and autonomic function. The least square mean change from baseline in the mNIS+7 score at 18 months was –6.0 ± 1.7 with patisiran treatment compared to 28.0 ± 2.6 for the placebo group ($p < 0.001$). Improvements were observed in secondary endpoints compared to placebo, such as Norfolk quality of life–DN score, modified body mass index, and 10-m walk test (54). Reported adverse events were consistent with those reported in the previous study, with peripheral edema and infusion-related reactions being the most common. An application for market authorization in the United States and European Union has been approved.

Eteplirsen

Eteplirsen is a 30-mer morpholino ASO (**Figure 3**) designed to bind to exon 51 of the dystrophin pre-mRNA and promote skipping of exon 51. In those DMD patients who have select mutations in upstream exons, skipping exon 51 produces a truncated but partially active dystrophin product (55). The first clinical study was a biochemical proof-of-concept study, in which eteplirsen was administered intramuscularly into the extensor digitorum brevis muscle and saline was injected into the contralateral muscle. An increase in dystrophin expression in biopsies taken from the injected muscle was demonstrated, with samples in the high-dose group achieving up to 32% of normal dystrophin expression (16).

This study was followed by two small studies evaluating different doses of eteplirsen (ranging from 0.5 to 50 mg/kg per week) administered as an intravenous infusion to DMD boys (44, 56). The first phase II study evaluated doses ranging from 0.5 to 20 mg/kg per week for 12 weeks (44). The drug was well tolerated with no safety concerns identified. A dose-dependent increase in dystrophin expression was demonstrated in muscle biopsies from treated subjects. Seven subjects in the higher-dose groups had an increase in the number of dystrophin fibers and a commensurate increase in the mean intensity staining for dystrophin, but expression was variable. A reduction in inflammatory infiltrates was also reported in those subjects with increased dystrophin expression. In a follow-up small (four subjects per cohort), controlled study, DMD boys were dosed weekly with 0, 30, or 50 mg/kg eteplirsen for 24 weeks by intravenous infusion (56). Subjects infused with placebo were switched to either 30 or 50 mg/kg eteplirsen at week 25 (two subjects each). Muscle biopsies were performed at baseline and week 48 for all subjects, and subjects in the 30 mg/kg dose group were also biopsied at week 24. The primary endpoints of this phase II study were distance walked in six minutes and dystrophin-positive fibers. There was no difference in the change in the six-minute walk test across the groups at 24 weeks. Modest increases in dystrophin expression were measured in both the 30 and 50 mg/kg dose groups at week 48, but the clinical significance of the small increase and technical methods used to measure dystrophin in this study were criticized by the US Food and Drug Administration (FDA) (57, 58). Although this small study was not designed as a pivotal study to support registration of the drug, the sponsor made the decision to file for market authorization based largely on data from the study. After significant delays, the FDA, in a controversial ruling, granted provisional approval (59) based on the long-term follow-up data from patients on this study and reanalysis of dystrophin expression using validated assays (60, 61).

Nusinersen

Spinal muscular atrophy (SMA) is a recessively inherited neuromuscular disease caused by mutations or deletions in *survival motor neuron* 1 (*SMN1*), a gene found on chromosome 5 (62). Patients with the most severe form of the disease, type 1 SMA, present with symptoms within the first few months of life, never gain the ability to sit, and generally succumb to their disease in their first year without continuous ventilatory support (63). In the less severe forms of the disease, patients gain the ability to sit but are unable to walk (type 2) or learn to walk but often lose this ability as teenagers or young adults (type 3).

The chromosomal region where *SMN1* resides has undergone an inverted duplication that contains at least four genes including *SMN2*. *SMN2* differs from *SMN1* by just a few nucleotides; one difference is a C-to-T transition within exon 7, which weakens the splice site. As a result, the majority of transcripts derived from *SMN2* skip exon 7, producing a truncated protein that is rapidly degraded. Approximately 15–20% of the transcripts from *SMN2* are properly spliced and produce a fully functional SMN protein. Therefore, the more copies of *SMN2* a patient has, the more SMN protein is produced, and the less severe the disease is. All SMA patients must have at least one copy of *SMN2*, with type 1 patients generally having two copies, type 2 patients 2–3 copies, and type 3 patients 3–4 copies (63). Based on the genotype–phenotype correlation, strategies to develop therapies are focused on increasing SMN protein production either through gene therapy (64) or through alteration of splicing of the SMN2 pre-mRNA to increase exon 7 inclusion using ASOs (17, 65).

Nusinersen is a uniformly modified MOE ASO, 18 nucleotides in length (**Figure 3**), designed to bind to a sequence present in intron 7 of *SMN2* (65). Nusinersen promotes dose-dependent increases in exon 7 inclusion in the SMN2 mRNA and increases SMN protein in transgenic mice expressing human *SMN2*. These increases have translated to improved survival and motor function (17, 65, 66). Because ASOs do not cross an intact blood–brain barrier, the drug was administered into the CSF. For human clinical studies, the drug was evaluated as an intrathecal injection by lumbar puncture. The initial clinical study of nusinersen was in type 2 and type 3 SMA subjects, which allowed evaluation of safety and tolerability of the intrathecally administered drug in a medically stable patient population (67). Based on encouraging safety and clinical signs, two open-label multiple-dose phase II studies were initiated, one in type 1 SMA patients and the other in types 2 and 3 (41). Even though the study of type 1 SMA patients was an open-label study, the results were unexpected. Gains in motor function and improved survival compared to the natural history were observed, as well as a demonstration of biochemical proof of drug mechanism (41).

Two sham controlled phase III studies were conducted, one in subjects predicted to develop type 1 SMA on the basis of *SMN2* gene copy number and age of disease onset and a second in subjects predicted to develop type 2 SMA on the basis of gene copy number, age of onset, and achievement of motor milestones (68, 69). Both clinical studies were stopped in response to a preplanned interim analysis in which improvements in clinical outcomes favored drug-treated subjects. All subjects in the study could enroll in an open-label study following the interim analysis. In both cases, the findings from the small open-label studies were replicated, demonstrating improvements in motor function in both patient populations and improved survival for type 1 SMA patients. In both studies, the incidence and severity of adverse events were similar for nusinersen and sham cohorts (68, 69).

Results from a study examining the effects of nusinersen treatment in SMA subjects who were administered the drug prior to symptom onset are even more exciting. In most cases, patients who were predicted to develop type 1 SMA on the basis of *SMN2* copy number are achieving

developmental milestones at appropriate intervals (70). These results support early identification and treatment of patients at risk of developing SMA through newborn screening.

Based on these data from the controlled study, the FDA and other regulatory agencies have approved nusinersen for the treatment of SMA. The leadership in the Neurology Division of the FDA highlighted the nusinersen development program as a model of how to successfully develop a drug for a rare disease (71). Nusinersen is the first drug approved for the treatment of SMA and is the first drug to demonstrate that increasing SMN protein production in patients with SMA can improve motor function, rather than merely stabilize the disease, as was expected when the clinical trials were started. Finally, it is the first intrathecally delivered antisense drug to be approved, validating this route of administration for other neurological diseases.

FUTURE PERSPECTIVE

Forty years after Paul Zamecnik and Mary Stephenson first published the concept of using synthetic oligonucleotide to inhibit viral replication by blocking translation of viral RNA (9, 10), we are beginning to see the idea become a reality. There have been many successes as well as disappointments over that 40-year period. As of this writing, there are six approved antisense drugs, one under regulatory review for approval, and several additional drugs in late stages of development (5). Nusinersen has been a commercial success, validating the commercial potential of ASO drugs. Based on these successes, ASO technology has become an important drug discovery platform for most major pharmaceutical companies.

It is important to recognize that ASO technology is still maturing. There are many opportunities to further improve the platform and create better antisense drugs. As an example, newer ASO drugs incorporating GalNAc conjugates to enhance delivery to the liver are showing 20- to 30-fold increases in potency for RNase H1-dependent oligonucleotides (72) and very potent siRNA drugs (73). This increase in potency allows less drug to be used, possibly reducing or eliminating some of the adverse effects observed with higher doses of ASOs, and supports less frequent dosing. The demonstration that targeted delivery of ASOs can dramatically improve their potency provides strong motivation to identify other ligands to enhance activity in other tissues. Investigations into the mechanism of cellular uptake of ASOs demonstrated that the majority of ASO in cells is localized in intracellular vesicles, which restrict access to the targeted RNA (74). There is more to learn about how ASOs are taken up into cells and escape from these endosomal/lysosomal vesicles. Improved knowledge could lead to ASO chemistry and designs that more efficiently escape these structures and potentially identify drugs that could enhance the intracellular availability of ASOs.

There are opportunities to further improve the safety and tolerability of ASOs. For example, the mechanism(s) by which some PS-modified ASOs reduce platelet counts warrants further investigations to help design safer drugs. As our knowledge of RNA regulatory mechanisms improves, it is likely that additional antisense mechanisms will be identified. Finally, there is opportunity to further improve patient convenience, such as injection/delivery devices and oral formulations. The demonstration that ASO drugs are producing major therapeutic benefits to patients who, up to now, have not had therapeutic options justifies continued investments to further improve the technology.

DISCLOSURE STATEMENT

The author is an employee of Ionis Pharmaceuticals, Inc., and receives salary and stock options from the company. Inotersen and nusinersen are drugs discovered by scientists at Ionis Pharmaceuticals and the company receives royalties on sales of the drugs.

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